ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

BIOMASS PRODUCTION FROM *SCHIZOCHYTRIUM SP.* AND ANALYSIS OF THE BIOMASS CONTENT

M.Sc. THESIS

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Molecular Biology – Genetics and Biotechnology Department

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SCHIZOCHYTRIUM SP. MİKROALGİNDEN BİYOKÜTLE ÜRETİMİ VE ÜRETİLEN BİYOKÜTLENİN İÇERİĞİNİN TAYİNİ

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MAYIS 2015

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To my family, my adviser and to my dear friends who always stands with me,

FOREWORD

This thesis examine one of the alternative lipid production proceses for edible nutraceutical lipids acquired from microalgae named as Schizochytrium sp. heterotrophic microalgae. It is proliferated, grown, filtered, dried and the content of the organism is analzed in order to quantify the lipid amount obtained.

The main part of the thesis was done in Istanbul Technical University in 2014. Some of the experiments were conducted in a company studying on microalgae production especially for nutraceutical lipids. I would like to thank Vitatis Biotechnology company for the guidance during my thesis.

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May 2015

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ABBREVIATIONS

DHA **:** Docosahexaenoic acid
EPA **:** Eicosapentaenoic acid
PUFA **:** Polyunsaturated fatty acid
LA **:** Linoleic acid
ALA **:** α-linoleic acid
LDL **:** Low-density lipoprotein
HDL **:** High-density lipoprotein
PCB **:** Polychlorinated biphenyl
DDT **:** Dichlorodiphenyltrichloroethane
FAO **:** Food and agriculture organization
ATCC **:** American Type Culture Collection
DMSO **:** Dimethyl sulfoxide
PC **:** Phycocyanin
PPP **:** Pentose phosphate pathway
TCA : Tricarboxylicacid

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BIOMASS PRODUCTION FROM *SCHIZOCHYTRIUM SP* AND ANALYSIS OF THE BIOMASS CONTENT

SUMMARY

Biotechnology has been working for the goods of livings in order to develop procceses and products for years. Human well-being takes the first place through these studies especially the production of essential metabolic molecules. Omega fatty acids are one of those significant molecules and getting more attraction at last decades. The process of obtaining these fatty acids from microalgae instead of cold marine fishes is a populer topic as well. There are many species metabolizing these omega fatty acids for example *Crypthecodinium cohnii*, *Schizochytrium sp* and *Nannochloropsis sp.* *Schizochytrium sp*. was known to belong fimgi family but then substituted to under Thraustochytrids family as heterekont algae with the help of modern genetic and molecular methods. This alga produce high amount of DHA, which is an essential fatty acid for our metabolism. The current source of this polyunsaturated fatty acid is cold marine fishes but this system has many deficiencies like not being sustainable and environmentally friendly.

In this study, all the experiments were conducted to produce a high amount of *Schizochytrium sp.* microalgae biomass and so to obtain fatty acids within this one cell organism. The aim of the study was to develop a sustainable, environmentally friendly, scalable and non-toxic process in order to growth *Schizochytrium sp.* to obtain omega fatty acids. There are heterotrophic and phototrophic algae metabolising high amount of lipids, proteins and carbohydrates. Phototrophic algae production was populer at the beginings because the sun energy and salty water were free sources in the production processes. But then it was realised that the amount of biomass and lipid content was very low. Also this system dependent to sun and other environmental factors. This means that system has negative points as the fish based systems. Therefore, the heterotrophic methods are designed and many microalgae species are isolated which are able to grow heterotrophically and can produce high amounts of lipids, proteins and carbohydrates.

In this study *Schizochytrium sp* was acquired from ATCC and then stock cultures were prepared, one for seed stock and the others for working stocks. Two-stage growth method was used, firstly the main stock was inoculated to a complex medium for 48 hours under 25^{o} C and then those cells were inoculated to defined mediums. Lastly, these cells were incubated for 48 hours and then the resulting content was centrifuged to obtain the biomass produced. This biomass was dryed then the resulting biomass is weighted. Then this biomass was smashed and subjected to hexane extraction in order to obtain the oil. Additionally, nile red staining also processed to quantify the total oil.

Results showed that a large-scale production method could be implemented with fermenter conditions to produce high amount of biomass and lipids.

SCHIZOCHYTRIUM SP MİKROALGİNDEN BİYOKÜTLE ÜRETİMİ VE ÜRETİLEN BİYOKÜTLENİN TAYİNİ

ÖZET

Bu çalışmada *Schizochytrium sp.* heterotrofik mikroalginin büyütülmesi, elde edilen biyokütle miktarının belirlenmesi ve hücre içindeki yağ oranının ölçülmesi üzerine çalışılmıştır. Tezin amacı son yıllarda giderek popüler konular arasına giren mikrolaglerden yağ asitleri ve biyodizel elde edilmesi gibi alternatif kaynaklar kullanılarak sürdürülebir, ölçeklendirilebilir, verimi yüksek, çevre dostu ve temiz bir üretim yöntemi oluşturmaktır. Bu amaçla ATCC (Amerika Birleşik Devletleri) firmasından alınan *Schizochytrium sp.* hücresi kullanılarak küçük ölçekte üretim denemeleri yapılmış ve elde edilen veriler literatürde yer alan bilgilerle karşılaştırılmıştır. *Schizochytrium sp.* ilk olarak fimgi ailesine mensup olarak sınıflandırılsa da daha sonra modern genetik ve moleküler yöntemlerle yapılan analizler sonrasında *Thraustochytrids* heterokont alg ailesine dahil edilmiştir. Daha çok deniz ekosisteminde yer alan organizma ile balıklar ve diğer deniz canlıları beslendiğinden bir anlamda insan besin zincirinin de bir parçası olduğu söylenebilir. DHA ve EPA tüketimine olan ilginin artmasıyla bu yağ asitlerini sentezleyen alternatif canlıların arayışına yönelik çalışmalar da hızla artmıştır. *Schizochytrium sp.* de bu anlamda ticari olarak kullanılan başlıca alg türlerinden biridir. Bunun en önemli sebepleri *Schizochytrium sp.*'nin heterotrofik ortamda büyümesini ve çoğalmasını kolaylaştıran özelliklerinin bulunmasıdır ve ayrıca organizmada uzun zincirli doymamış yağ asitlerinin sentezini sağlayan ve oksijenden bağımsız aktivite gösteren bir enzim kompleksinin varlığıdır. Bu sayede oksijen kısıtlı olduğunda bile mikroalg yağ sentezine devam edebilmekte ve hücre içinde biriktirmeyi sürdürmektedir. Tek hücreli bir canlı olduğu için de hücre içeriğinde sentezlenen moleküllerin saflaştırılmasına olanak sağlamaktadır. Kontrol mekanizması yüksek olan fermentörlerde heterotrofik koşullar altında üretimi diğer mikro alg türlerine göre daha kolay olan *Schizochytrium sp.* algi çok sayıda sektöre alternatif molekül ve ürün sağlamak amacıyla çalışılmaktadır. Ortamdaki çözünmüş oksijen konsantrasyonu, tuzluluk, karbon ve azot miktarı, karbon ve azot kaynaklarının türleri ve sıcaklık parametleri optimize edildiğinde sürekli ve verimli olarak üretime elverişli bir sistemin oluşturulması üzerine yapılan çalışmalar sonucunda *Schizochytrium sp.* 'den DHA üretimi için sürdürülebilir sistemler kurulmuş ve pazarda ürünleri hizmete sunulmuştur.

DHA ve EPA gibi insan sağlığına faydalı ve esansiyel olan uzun zincirli yağ asitlerinin elde edilmesinin yanısıra, protein ve karbonhidrat gibi organik moleküllerin de ticari hacimlerde üretilebilmesine yönelik *Schizochytrium sp*. ile yapılan çalışmalar devam etmektedir. Hücre fizyolojisinde yağların çok farklı yerlerde ve rollerde görev aldığı bilinmektedir. Bu amaçla çok sayıda çalışma da yapılmaktadır. Bir hücrenin yağ içeriği çevresel koşullara bağlı olarak değişebilmekte ve bu değişimler hücrenin zarında çeşitli modifikasyonlara yol

açabilmektedir. Yağ asitleri farklı sayıda karbon atomundan oluşan hidrokarbon zincirlerini içeren düz zincirli monokarboksilik asitlerdir. Bazı yağ asitlerinin hidrokarbon zincirleri hiç çift bağ içermez ve doymuş yağ asitleri olarak isimlendirilirken bazılarının hidrokarbon zincirleri bir veya daha fazla çift bağ içerirler ve doymamış yağ asidi olarak isimlendirilirler. Bu yağ asitlerinin metabolik aktiviteleri de karbon sayısı ile birlikte içerdikleri karbon çift bağının sayısına ve yerine bağlı olarak değişebilmektedir. Tezin çalışma konusunu oluşturan DHA (Docosahexaenoic Acid) ve EPA (Eicosapentaenoic acid) omega-3 yağ asitleri uzun zincirli doymamış yağ asitleri grubuna girmektedir. Esensiyel yağ asitleri olmaları nedeniyle, insan vücudunda bu yağ asitleri üretilemediğinden beslenme yoluyla tüketilen gıdalardan alınmaktadır. Bunlar arasında en temel yeri balıklar ve kuru gıdalar oluşturmaktadr. DHA vücudumuzda birçok önemli fonksiyonun sağlıklı bir şekilde gerçekleşebilmesi için oldukça önemli bir yağ asididir. Hamilelik süresince bebeklerin ilk altı aylık döneminde göz, beyin ve sinir sisteminin gelişiminde DHA önemli bir rol oynamaktadır. Vücudumuzda beyin, sinir sistemi, sperm, serebral korteks ve retina başta olmak üzere birçok önemli fizyolojik noktanın temel yapıtaşıdır. Yetişkinler özellikle balık ve benzeri ürünlerle beslenerek, bebekler ise sadece anne sütü alımıyla bu yağ asitlerini temin edebilmektedirler. Bunların yanısıra kanserden kardiyovasküler hastalıkların önlenmesine kadar daha birçok alanda faydası bulunduğu, yapılan çalışmalarla gösterilmiştir. Omega-3 yağ asitlerinin gen düzeyinde de etkilerinin bulunduğu gösterilmiştir. Bazı gen ekpresyonlarında bu yağ asitlerinin rol aldığı ileri sürülmektedir. Doymuş yağ asitlerinin hidrokarbon zincirleri hiç çift karbon bağı içermediğinden, çok sıkı yapılar oluşturabilmektedirler. Eğer bu çok sıkı paketlenmiş yapılar kanda çoğalırlarsa LDL denilen ve halk dilinde kötü kolesterol olarak bilinen moleküllerin kandaki oranı yükselir ve damar yolu problemlerine yol açabilir. Doymamış yağ asitleri ise yapılarındaki çift bağ nedeniyle çok sıkı paketlenmiş yapılar oluşturamadıklarından kanda HDL olarak bilinen iyi kolesterolün çoğalmasını sağlamaktadırlar. İyi kolesteroller hücre zarında geçirgenliği arttırdığı gibi kötü kolesterolün de karaciğere taşınıp vücuttan atılmasını sağlamaktadırlar.

Omega yağ asitlerinin beslenmedeki önemlerinin anlaşılmasına paralel olarak yıldan yıla tüketimlerinin de artması ciddi bir pazar ve ticari hacim oluşturmaktadır.Özellikle ticari değeri ve alternatif temiz enerji kaynağı olarak biyodizel üretimi çalışmaları için kullanılan mikrolgler bu anlamda geleceğin enerji kaynağı olarak da gösterilmektedir. İnsanların çok ciddi hastalıklara yakalanma oranlarının arttığı son yıllarda bu tarz sağlıklı ürünlere olan ilgi giderek artmaktadır ve özellikle omega yağlarının tüketimi çok hızlı artış göstermektedir. Ancak omega yağ asitlerinin temel kaynağının soğuk deniz balıkları olduğu düşünülürse giderek artan bu talebi uzun vadede karşılayamayacağı öngörülebilmektedir. Hem DHA içerikli paketlenmiş gıdaların geliştirilmesi hem de ilaç ve nütrasotik sanayiinde sürekliliği olan ürünlerin elde edilebilmesi için farklı kaynak arayışları için yola çıkılmıştır. Balıklardan elde edilen ürünler çevresel faktörlere çok bağımlıdır. Mevsimsel balık stoklarında yaşanan değişimler, denizlerdeki kirlilik oranları, üretilen ürünün tat ve kokusundan duyulan rahatsızlık gibi dezavantajlar nedeniyle balık yerine alglerden omega üretmek çok daha avantajlı bir yöntem olarak hızla gelişmektedir. Elde edilen ürünlerin vejetaryanlar tarafından kullanılamaması da diğer bir sorun olarak karşımıza çıkmaktadır. Günümüzde yoğun olarak çalışılan farklı mikroalg türlerinin karşılaşılan bu tür sorunlara alternatif çözüm sunacağı düşünülmektedir. Gelişen biyolojik yöntemlerin ve teknolojinin yardımıyla hızla artan talebi karşılayabilmek amacıyla çalışmalar yapılmaktadır. Farklı mikroalg

türleri ve kültür yöntemleri kullanılarak verimli sonuçlar elde edilmektedir Hem fototrofik hem de heterotrofik olarak üretilebilen bu canlıların son yıllarda hızla gelişen genetik ve moleküler biyolojik yöntemlerle verimlerinin yükseltilmesi üzerine de çalışmalar yapılmaktadır. Özellikle tuzlu su kaynağı ve güneşin yılın uzun bir döneminde aydınlattığı yerlerde fototrofik mikrolagler kullanılarak maliyetleri düşük üretim çalışmaları yapılmaktadır. Açık alanlarda tasarım olarak yüzey alanını arttıran ince cam ya da ışığı geçiren alternatif bir malzemeyle yapılmış borular kullanılarak büyütülen mikrolagler yağ asidi eldesi için kullanılmaktadır. Büyütülen mikroalgler önce filtre edilmekte, sonra kurutulmakta ve daha sonra da içeriği saflaştırılarak hücre içinde metabolize edilen yağlar ayrıştırılmaktadır. Bu üretim yönteminin dezavantajı olarak mikrolaglerin güneş ışığında ve çevresel faktörlere bağlı olarak düşük verimde biyokütle oluşturması gösterilmektedir. Çevresel faktörlere bağlı bir üretim yöntemi balıklardan elde edilen ürünlerin taşıdığı riskleri taşımaktadır. Bu nedenle heterotrofik yöntemlerle üretilebilen ve fermentör gibi özellikleri ve çalışma prensipleri uzun yıllardır deneyimlenen kapalı sistemlerde üretim denemeleri daha başarılı sonuçlar vermiştir. Ülkemizde de aynı amaca yönelik çalışmalar yürütülmektedir. *Schizochytrium sp.* heterotrofik ortamda temel karbon, azot ve tuz kaynakları kullanılarak büyütülebilen bir organizmadır. Heterotrofik koşullarda yağ metabolizmasının %70'lere ulaştığı bilinmektedir. Bu oranda yağ elde edilebilmesi için bilinen kaynaklardan anlaşılabildiği üzere hücrenin büyütülürken azot kısıtlamasına tabi tutulması gerekmektedir. Bu aşamada hücre ATP sentez metabolizması yavaşladığından hayatta kalabilme güdüsüyle metabolik olarak yağ sentezlemeye başlamaktadır. Bu çalışmada *Schizochytrium sp*. kullanılarak yüksek verimde biyokütle üretimi ve üretilen biyokütlenin içeriğinin tespit edilmesini amaçlayan deneysel tasarımlar oluşturulmuştur. Farklı miktarlarda diamonyum tartarat kullanılarak büyütülen *Schizochytrium sp.* mikroalginden elde edilen biyokütle miktarı ölçülmüş ve içeriğindeki yağ oranı belirlenmiştir. Öncelikle kök ve çalışma stokları olarak hazırlanan hücreler sıvı azot tankında saklanmıştır. Daha sonra iki aşamalı büyütme yöntemiyle erlenlere ekimler yapılmış ve 48 saat boyunca 25 ºC`de çalkalamalı inkübatörde muhafaza edilmiştir. Birinci aşamada sıvı azot tankından alınan çalışma stoklarından kompleks besiyerine ekim yapılarak hücrelerin çoğalması sağlanmıştır. İkinci aşamada ise çoğaltılan bu hücrelerden sıvı besiyeri ortamına ekim yapılmıştır. 48 saat inkübe edilen hücreler daha sonra elde edilen biyokütlenin besiyeri ortamından uzaklaştırılarak miktarının belirlenmesi için santrifüj yardımıyla filtrelenmiştir. Filtrelenen biyokütle 3 cm yarıçapında filtre kağıtlarına ince tabakalar halinde serilerek vakumlu bir kurutucuda kurutmaya alınmıştır. Kurutulan besiyeri hassas terazi vasıtasıyla tartılarak elde edilen kurutulmuş biyokütlenin miktarı tayin edilmiştir. Elde edilen yağ miktarının belirlenmesi için nile red boyama yöntemi ve hegzan ekstraksiyonu kullanılmıştır.

Gelişen ve hızla değişen sağlıksız beslenme ve yaşam koşulları değerlendirildiğinde insanların ve hayvanların beslenmelerine daha özen göstermesi gerektiği önem kazanmaktadır. Bu amaçla tüketimi artan omega-3 ve omega-6 yağ asitlerinin üretimlerini sürdürülebilir ve bitkisel kaynaklı üretime elverişli şekilde geliştirmek kaçınılmaz olmuştur. Yapılan büyük ölçekte çalışmalar ve bu tezde yürütülen deneyler göstermektedir ki *Schizochytrium sp.* mikroalginden elde edilen yağ miktarları geliştirilebilecek üretim yöntemlerine elverişlidir.

1. INTRODUCTION

Biotechnological and interdisciplinary studies are proceed searching for the new developments to put into practice for all livings. Human being is taking the first place and health concerns are prioritized so the essential metabolic requisites for our metabolism is one of the popular topics through all. For example DHA production for supplemental feeding is getting larger and larger since 2000s (Harun et al., 2010). The use of the microalgae for the production of food suplemets, lipids, enzymes, polymers and toxins has been studied. Some of them are achieved by cultivating microalgae in different mediums and substrates (Perez-Garcia, O et al., 2011) *Schizochytrium sp*. is a well-known marine microalga that is improved as a source of docosahexaenoic acid (DHA, C22:6 (omega-3). In this study, *Schizochytrium Sp*. was acquired from ATCC and stocks of the cells are prepared. And then it was proliferated in different mediums containing different nitrogen amounts and the content of the biomass produced was analyzed. The experiments awere conducted especially for lipids DHA (docosahexaenoic acid) and EPA (eicosapentaenoic acid) accumulated in *Schizochytrium Sp.* which are products of a multi-subunit polyunsaturated fatty acid (PUFA) synthase.

1.1. Classification of Organism and General Features

Schizochytrium sp. was placed in the fimgi family first because of their heterotrophic nature and resemblance to the *Chytrids* (Sparrow, 1936). However, this organism was classified in *Thraustochytrids* as heterokont algae (Cavalier-Smith et al., 1994) by using modern molecular biology tools. *Schizochytrium sp.* occurs widely in marine environments. This organism can be considered as a component of the human food chain because fish and other marine animals feed on *Schizochytrium sp*. micro algae (Figure 1.1).

The increased attention to support human and animal diets with omega -3 fatty acids resulted an increase on the researches searching for alternatives to fish oil. Increased desire to supplement human and animal diets with omega-3 fatty acids

such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has increased the search for alternatives to fish oil. *Schizochytrium sp*. is one of several single cell organisms that produce oils rich in polyunsaturated fatty acids (PUFA) including DHA and EPA. Production of *Schizochytrium sp* have some advantages such as higher oxidative stability, lower purification costs, production from raw and sustainable materials and lastly constant product quality (Ratledge at al., 2004).

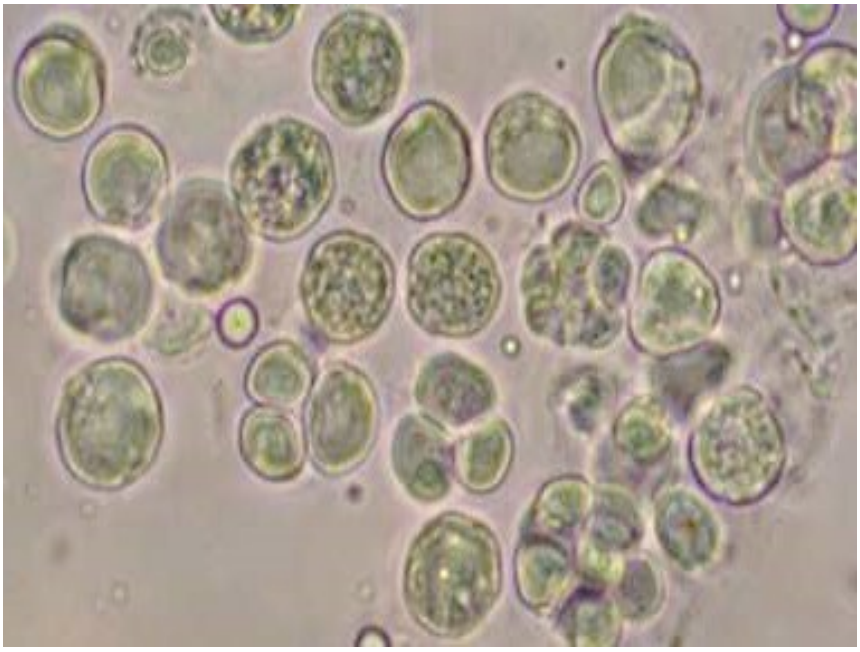

Figure 1.1 : Microscopic view of *Schizochytrium sp.* cells.

Schizochytrium sp. is one of the most popular commercial strain because it has a PUFA synthase complex which has an O_2-independent activity (Jakobsen et al. 2008). The lipid content of *Schizochytrium* could be increased under oxygen limitation conditions which may effect the activity of PUFA synthase enzyme complex. A two-stage growth trial of *Schizochytrium* showed that shifting dissolved oxygen level in shake flask scale was also investigated and DHA content was increased (Chi et al., 2009). These studies have also demonstrated that used dissolved oxygen cannot be easily applied to the large-scale processes. The two-stage growth study require further investigation to be applicable in industrial production.

1.2. Importance of Fatty Acids for Organisms

Lipids are defined as insoluble molecules in water and their biological functions as diverse as their chemistry. Many studies have been carried out to specify their biological roles in microbial cells. The lipid compositions of microorganisms vary

depending on different environmental conditions (Chihib, 2005) which may alter the physical characteristics of membranes such as fluidity, integrity and functionality of the membrane (Russell, 1984).

Fatty acids consist of hydrocarbon chains including a carboxyl group and a methyl group at two different ends. The metabolic activity of fatty acids is influenced by its length of carbon chain and also the number and position of double bonds. Fatty acids are differ having carbon-carbon double bonds. Unsaturated fatty acids have one or more carbon-carbon double bond but saturated fatty acids do not have any double bond between carbon atoms (P. C. Calder, 2008). If an unsaturated fatty acid contains two or more double bonds, unsaturated fatty acids are said to be PUFA. Omega-3 (n-3) and omega-6 (n-6) are two types of of PUFAs based on the location of the last double bond. The human body can produce fatty acids but two of the essential fatty acids namely linoleic acid (LA, C18:2n-6) and α-linoleic acid (ALA, C18:3n-3) which are are the basic members of PUFA cannot be synthesized (Wall et al., 2010). Saturated fatty acids are found in meats, butter and dairy products and they are solid at room temperature. However, unsaturated fatty acids are found in vegetable oils and they are liquid at room temperature. Because of their chemical differences in structure, saturated fatty acids and unsaturated fatty acids have different effects in our body (Aiguo, W. et al., 2004). Functions of essential fatty acids can be listed as blood pressure regulation, blood clotting, correct development and functioning of the brain and nervous system. DHA is required for the development of brains, eyes and nervous systems during the first six months of pregnancy because it is primary structural source of human brain, skin, sperm, cerebral cortex and retina. It can be acquired via maternal milk and fish oil (Guesnet, 2011). Like humans, also the other mammals obtain omega-3 fatty acids from dietary resources (Certik and Shimizu, 1999). All over the world, the main and commercial sources for these fatty acids are cold-water marine fish. They are rich in DHA so commercial importance had raised during the past decades. Nevertheless, because of the reasons specified at the begininhs of this section, some marine microalgae such as dinoflagellates and species containing high DHA are became more attracting for fatty acid production (Kirk E. Apt and Behrens, 1999). It is clear DHA is need to prevent human diseases such as cancer, cardiovascular disease (Lee and Lip, 2003), Alzheimer's disease and schizophrenia. Studies illustrates that they have effects on

gene level also. Omega–3 fatty acids play an important role to control the expression of specific genes in human body (Sessler and Ntambi, 1998). As we also know that, the metabolism of dopamine and serotonin in brain also can be affected negatively by the low level of DHA (Innis, 2007). Fully saturated fatty acids are packed together whereas unsaturated fatty acids are not packed together as tightly as saturated fatty acids due to several kinks that are forced by a cis double bond in the hydrocarbon chain. If these tightly packed saturated fatty acids enter the blood stream, the levels of low-density lipoprotein (LDL) cholesterol known as "bad" cholesterol are increased and artery problems occurs (Figure 1.2). On the contrary, unsaturated fatty acids are able to increase levels of "good" cholesterol and do not increase "bad" cholesterol. They are known as high-density lipoprotein (HDL) cholesterol and they are able to grab LDL and transfer them to the liver. Then the LDL is broken down and removed from the body (McKenney 2007).

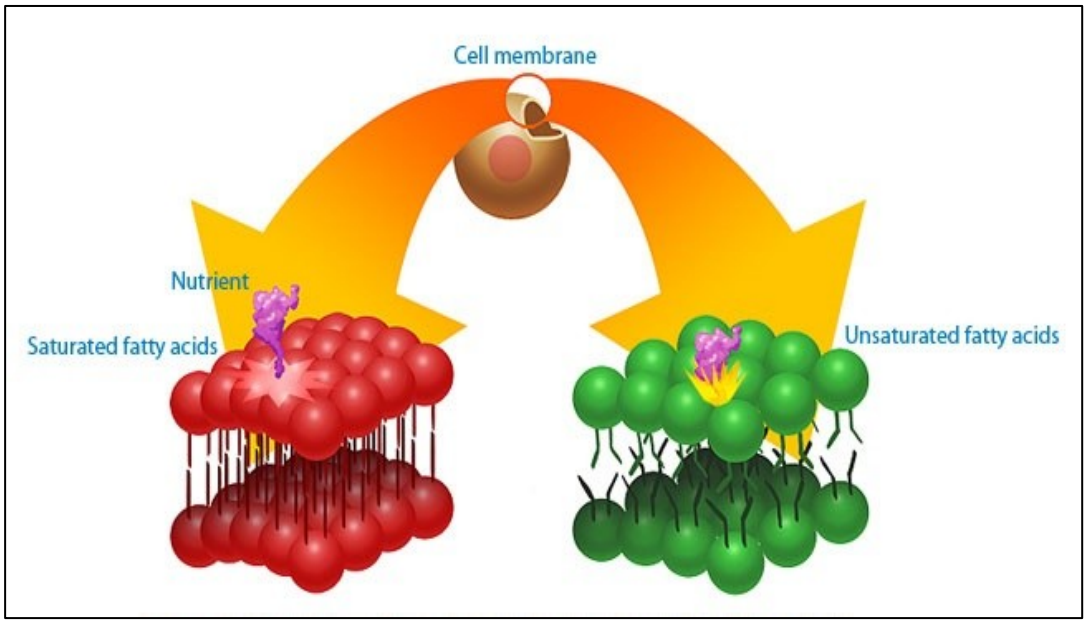

Figure 1.2: Importance of fatty acids on cell membrane.

Additionally, there are some other important long-chain polyunsaturated fatty acids essential for human body. They are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and they also have important roles in immune regulation and inflammation (P. C. Calder, 2009). We obtain some of them from our daily eating habits. For example, the main dietary sources of linoleic acid include plant oils such as sunflower, safflower, and corn oils (Table 1.1), but they are also present in cereals, animal fat, and whole grain bread. Rich dietary sources of ALA

(alpha linolnic acid) include green leafy vegetables, flaxseed, and rapeseed oils (Table 1.1).

Table 1.1: Examples rich in fatty acids (E. Patterson et all, 2012).

FAT TYPE	Linoleic Acid	α Linoleic Acid	Arachidonic acid	Docosahexaenoic acid + Eicosapentaenoic acid
SATURATED			1070	
Lard	8600	1000		
Butter fat	2300	1400		
Coconut oil	1400			
Beef tallow	80			
UNSATURATED				
1-Monounsaturated				
Peanut oil	23900			
Pecans	20600	1000		
Almonds	9860	260		
Olive oil	8000	950		
Avocado	1970			
2-Polyunsaturated				
Omega-6				
Safflower oil	74000	470		
Sunflower oil	60200	500		
Soybean oil	53400	7600		
Corn oil	50000	900		
Cotton seed oil	47800	1000		
Walnut	34100	6800	590	
Brazil nut	24900			
Omega-3				
Linseed oil	13400	55300		
Canola oil	19100	8600		
Salmon	440	550	300	1200
Tuna	260	270	280	400
Herring	150	62	37	1700
Trout	74		30	500
Cod	4	2	3	300

During the last decades, in our dietary life some radical nutritional changes have been occurred including increased levels of fatty acid consumption. Today, developed countries have also been classified by an increase in consumption of saturated fatty acids and trans-fatty acid (Olivier et al, 2011).

1.3. Commercial Potential

Commercial sources of omega-3 fatty acids including flaxseed oil, fish oil, and some fungi and algae oils, have been identified and recorded (Bajpai et al., 1991; Kendrick and Ratledge, 1992).

Global needs illustrate that fish oil alone is not expected to meet long-term large requirements of DHA by food, nutraceutical and pharmaceutical industries. Fish as a source of DHA has limitations such as low DHA content, variable DHA content depending on the environmental conditions, unpleasant odor and the presence of other undesirable PUFA. Currently, fish such as salmon, mullet and mackerel are the first hand source of EPA and DHA for human consumption (Gunstone FD., 1996) But there is a pity that global revenues have been diminishing and the stocks have been increasing exponentially since the 1950s (Worm B, et al., 2006). Above this, the pollution risk for world fish habitat is a crucial threat because the presence of chemical contaminants such as mercury and plumbum in fish oil can be harmful for consumers (Mahaffey KR, et al., 2008). In addition, fish oil has some other deficiencies for customers as it is not suitable for vegetarians and the odor makes it unattractive.

For these reasons a variety of alternative sources for EPA and DHA such as bacteria, fungi, plants and microalgae are currently being explored for commercial production. Some of them are profitable but some of them are not. In addition, the production processes are different and unsuitable for sustainable and long-term production. For example plants need cultivable land, have long growth period and have no enzymatic activity for producing long chain EPA and DHA, fungi need organic carbon sources and has long growth periods also. Currently microalgae are the primer EPA and DHA producers in the marine food chain. Because they can naturally grow fast under autotrophic, mixotrophic and heterotrophic culture conditions (Li Y, et al., 2009).

On the other hand, the issue has another point of view considering the rising human population. Fish and fish oils rich in EPA, DHA and other long chain fatty acids are currently recommended as primary sources of those. Consumption of these long chain fatty acids from traditional sources like fish has declined due to increased human population with disproportionately lower fish catches and variability of fish fatty acid profiles that depends on fish species, types and location of fish harvest

(Ackman and Ratnayake, 1990). The presence of toxic chemicals such as polychlorinated biphenyl (PCB) found in almost all fish (Addison and Ackman, 1974), DDT (dichlorodiphenyltrichloroethane) products and heavy metals in amounts exceeding food and agriculture organization (FAO) regulated limits of < 0.l pg/g for copper and arsenic in fish makes fish a less desirable source of EPA and DHA (Ackman and Ratnayake, 1990).

Consumption of EPA and DHA from new food sources has rapidly increased to meet dietary shortages of EPA and DHA so as stated several new sources of EPA and DHA, such as microalgae, have been identified (Kinsella, 1990). As a source of EPA and DHA, *Schizochytrium sp*. microalgae found in cold-water marine environments is now being produced commercially. Dried *Schizochytrium sp*. microalgae is used as poultry and animal feed ingredient to produce eggs, broilers, pork and beef enriched with DHA (Kinsella, 1990, Abril and Barclay, 1998).

Consequently, the search for alternate sources of DHA from microbial sources was initiated and production of *Schizochytrium Sp*. using fed batch technology has been recognized as a good source of DHA (Yaguchi et al., 1997).

This study has two main goals: first to increase the amount of biomass production and the oil amount in the biomass and second to analyze how different nitrogen sources will affect the total fatty acid amount of organism. Despite the high diversity of microalgae and cyanobacteria, these organisms are not widely used industrially compared to many other types of microorganisms. Most microalgae and cyanobacteria depend on externally supplied light, which makes large-scale cultivation technically difficult and expensive. *Schizochytrium Sp*, *Spirulina, Dunaliella, and Chlorella* are successfully cultivated in inexpensive, open pond systems by using the sun as the light source, but typically only at biomass dry weight concentrations of 0.5–10 g L^{-1} (Lee Y.J., 2001). These concentration levels are very low compared to those found in industrial cultures of heterotrophic bacteria and yeasts, which may be grown to biomass dry weight concentrations greater than 100 g L^{-1} (Chauhan et al., 1999; van Hoek et al., 2000). Low growth rate biomass production in light-dependent algal cultures grown in open ponds or enclosed photo bioreactors are only around 0.2 - 4.3 g L^{-1} day^{-1} (Lee Y., 2001). This is substantially lower productivity than it is obtained in cultures of heterotrophic microorganisms. Only a few of microalgal species are well adapted for heterotrophic growth (Chen,

1996) and only a very few species, like the green alga *Chlorella spp*. the dinoflagellate *Crypthecodinium cohnii* (de Swaaf et al., 2003) and the diatom *Nitzshia laevis* are cultured heterotrophically in high cell density cultures at biomass levels of 50 g L^{-1} and above (Wen and Chen, 2003). Microalgae are the primary producers all over the world that they photosynthetically convert light energy and carbon dioxide (CO_2) into biomass such as carbohydrates, proteins and lipids (Becker, 2007). During microalgal production, the limitation of nutrients or light restrain the increase of biomass. Under high nutrient supply conditions algae proliferation occur at very high cell density levels (Sellner et al, 2003). Under nutrient limiting environments except light, accumulation of photosynthetic bio products such as lipids and carbohydrates can be increased. This is a kind of survival mechanism that storage products in order to be alive under the stressful growth limiting circumstances (Anderson et al, 2002). In extreme cases sometimes this can lead anaerobic life, causing the death of plants and animals but interestingly this process is also believed to be the key factor for anoxic events that led to mineral oil deposition (Schenk et all, 2008). Importantly, microalgae are also the primary producers of EPA and DHA that are accumulated through the various nutritional levels. Changes in microalgal lipid content are carried on through the food chain for example zooplanktons and some fish so this subsequently affects the accumulation of EPA and DHA fatty acids in higher organisms and humans (Brown, 2002). Finally, lipid production in microalgae play a key role in maintaining the world's aquatic food resources.

1.3.1. Biodiesel applications from algae

Biofuels from microalgae is an attractive option for microalgae biotechnology. Biofuels obtained from microalgae is an impressive method for microalgae biotechnology since it is the most effective way with respect to all the others because crude oil has a high price. Also, biodiesel has a lot of benefits for machines and the environment since it is convenient to combine with petroleum-based diesel fuel (Li et al., 2008). Recently, majority of the biodiesel is produced from vegetable oils for example canola, sunflower and palm obtaining the long-chain fatty acids (LCFA). However, this means periodical production limitation and this is a major limitation and sufficient reason to search for other sources of LCFA. Therefore, these reasons lead to find other sources of LCFA. Producing biodiesel is not sustainable because

available method has some problem such as food supply and food security. Due to the cheaper heterotrophic cultivation, some microalgae species can be used to enlarge lipid production and microalgae cultivation so biodiesel derived from microalgae is an impressive and applicable method thus some species can be improved genetically to produce more lipid content (Song et al., 2008). This means that microalgae offer the greatest opportunities compared to oilseed crops in the longer term. Productivity of many microalgae are quite high that, under heterotrophic conditions, oil content of many microalgae strains is around 80% (Khan et al., 2009; Huang et al., 2010). Additionally, their processes for production and obtaining biofuel, are economic, well-known technology and environmentally sustainable. Furthermore, their production is not seasonal and has advantages to harvest daily products. Current mass production of microalgae requires significantly less land area than crop-based biofuels and releases fewer pollutants to the environment (Chisti, 2008).

C. protothecoides is a convenient microalga for biodiesel production because it uses organic carbon sources in a heterotrophic manner. Quantities of lipids can be acquired up to 50% of its dry weight by using this species (Mata et al., 2010; Sivakumar et al., 2010). Using *C. protothecoides* biodiesel was produced from hydrolysate of the Jerusalem artichoke tuber under heterotrophic conditions, with significant cost reduction (Chen et al., 2012). This biodiesel was comparable to oil-based diesel and complies with the US Standard for Biodiesel (Miao and Wu, 2006). Glycerol has a feasible carbon reserve to obtain valuable products in a heterotrophic process. It is also an inexpensive carbon generated as a by-product of biodiesel fuel production. So, this crude glycerol need to be converted into higher-value products and there are development areas to discover new process for this conversion. Because carbon atoms in glycerol are highly reduced so, the fuel and other reduced chemicals can be generated much more than obtained from glucose (Yazdani and Gonzalez, 2007).

To sum up, economic parameters illustrate the commercial potential of this source, produced biodiesel by heterotrophic microalgae is new study era to show economical potential of this source with limited knowledge (Spolaore, 2006). Due to the inexpensive carbon sources containing lots of long-chain fatty acids and microalgae metabolism, this is an impressive research area for future.

1.4. Alternative Bioproducts From Algae

The alternative compounds synthesized by microalgae also have several applications. For example there are some algae species having high-protein content so they can be considered as a nonconventional source of proteins (Sánchez et al., 2008). On the other hand nutritional value of those proteins and availability of the amino acids should be examined further (Borowitzka, 2013). Carbohydrates have more potential through these compounds. Since, microalgal polysaccharides like agar and others are used in variety of fields in industry due to their colloidal and other chemical properties. The omega-3 and omega-6 families; lipids and fatty acids from microalgae, have more interest because of their health effectiveness (Borowitzka, 2013). The class of other important compounds obtained from microalgal and cyanobacterial cultures are photosynthetic pigments particularly carotenoids and phycobiliproteins.

The microalgae are known to be extremely a diverse collection of organisms and this variety is not yet explored in details. This evolutionary diversity also means a huge diversity in the chemical composition of these organisms. They are extremely attractive for new bio products and potential commercial sources of a wide range of biomolecules. This possible potential for new valuable chemicals and other products recently attracting huge interest (Stephens et al., 2010). For example, algal oils for biofuels are valuable products whereas they are not commercialized due to high cost of their production. Thus, algal oils have more catch potential to produce. Additionally, the remaining biomass can be sold as animal feed, the value of that catch might be more. Actually, researches show that the development of commercial products from microalgae are not new. The earliest usage of microalgae as a source of lipids is in 1942s and carotenoids 1964. According to Tamiya (1957), “The first commercialized microalgae were Chlorella and Spirulina as ‘health food’ in Japan, Taiwan and Mexico”. Following this, β-carotene from *Dunaliella salina* was commercialized (Chen, 1996). Astaxanthin from *Haematococcus pluvialis* and docosahexaenoic acid from *Crypthecodinium cohnii* are other well-known industrial examples (Kyle, 2005). Additionally microalgae are not the only source of those valuable products actually. They can also be produced by chemical synthesis or from other organisms such as fungi, bacteria, higher plants naturally (Demain, 2009). On the other hand, the production costs can be lowered. There is a good example for

these products are the vitamins. Algae based vitamins are currently cannot compete in the market though they have been suggested as commercial sources such as vitamins C, E and B12 (Borowitzka, 2013; Doncheck et al., 1996).

Pigment synthesis is normally low in heterotrophic grown microalgae and therefore, heterotrophic cultures are generally not well suited for these types of products (Chen, 1996). However, some strains of the unicellular, acidophilic red alga *Galdieria sulphuraria* retain their photosynthetic ability when grown heterotrophically in darkness (Gross and Schnarrenberger, 1995; Marquardt, 1998). A major pigment in this alga is phycocyanin (PC), which is used as a fluorescent marker in diagnostic histochemistry (Glazer, 1994; Glazer and Stryer, 1984), and as a dye in foods and cosmetics (Benemann et al., 1992). PC may also have potential as a therapeutic agent.

Through all those algae, our organism *Schizochytrium sp*. have been developed in fed-batch cultures during our study. The effect of nitrogen source in the heterotrophic production of biomass has been investigated. According to researches, optimal growth conditions of *Schizochytrium sp* are optimized and under these conditions the risk of contamination by other organisms is minimal and *Schizochytrium sp*. is able to utilize a large variety of biomass sources.

Many processes are designed during decades for the commercial and industrial fed-batch biomass productions. The cell biomass is proliferated and growth in batch cultures. After that the biomass and the culture is separated and the cell biomass was determined by centrifuging the cell suspension, washing and drying. Total lipid was extracted via different chemical processes like chloroform: methanol (2:1) extraction, supercritical carbon dioxide separation and hexane extraction that is used for organic molecules generally (Spolaore, 2006). The extracted lipids were then analyzed in order to obtain how much of the oil is DHA. With a basic way, cultures were grown in Erlenmeyer flasks with basal medium with the experimental carbon and nitrogen sources. The initial pH of the culture is controlled with buffer (potassium dihydrogen phosphate–sodium hydroxide). EPA and DHA are highly unsaturated fatty acids and are susceptible to autoxidation (Gunstone, 2012). Ethoxyquin, a synthetic, lipophilic antioxidant is currently added in vitro to *Schizo*. to stabilize PUFA in the algal oil and to extend shelf-life of the dried *Schizo.* (Barclay et al., 1994, 1998).

To further understand the physiological and biochemical characteristics of this marine fungal, it is necessary to analyze cellular fatty acids' behaviors under different cultivating conditions. Although the fatty acid compositions of *Schizochytrium* at different developmental stages and nutrient levels have been reported recently, the fatty acid profile at different temperatures and salinities has not been studied (Jiang et al., 2004).

1.5. Parameters Effecting the Biomass and Oil Production

Successful cultivating process requires specific environmental conditions and these conditions vary from specie to specie. The main parameters can be listed as temperature, aeration, nutrient composition and amount, salinity and mixing conditions.

1.5.1. Temperature

With a general knowledge of algae proliferation parallel to the exponential temperature rise until an optimum level is accessed. This is more important for open pond cultures because the control mechanism for temperature is limited and regulated by atmosphere and humidity. The temperature fluctuation in ambient conditions affect the productivity of algae. If optimal culture temperatures cannot be achieved until the organism starts to metabolize organic molecules it results a lack of synchronization problem within the organism.

While the temperature is fixed below the optimal conditions it does not kill the algae as generally known except the freezing conditions. *Schizochytrium sp.* is grown under a temperature range between 24-28^{o} C and optimally 25^{o}C but there has been no significance difference through these temperature conditions as the results illustrates.

1.5.2. Mixing and aeration

The leak of light and gases through the medium mixture is important for both photosynthetic and heterotrophic microalgae production. Erlen flasks are incubated under 250 rpm conditions during our studies. The aeration conditions adjusted steady and environmental parameters are blocked for any fluctuation with a close incubator. Temperature differences are also blocked due to importantance on biomass growth.

During the 48-hour incubation period, mediums are handled under stable mixing conditions in order to achieve truly processed lag, log and stationary phases.

1.5.3. Carbon metabolism

As it is known for many other microbial species, glucose is the common carbon source for heterotrophic cultures of microalgae as well. According to experiments processed with any other substrate such as sugars, sugar alcohols, sugar phosphates, organic acids and monohydric alcohols higher growth rate are achieved in the presence ofglucose (Griffiths et al., 1993). The reason behind is probably the possession of more energy content per mole glucose compared with other substrates (Boyle and Morgan, 2009). In details, glucose-6-phosphate, which is available for storage, cell synthesis and respiration in cell, is obtained via oxidation of glucose with a phosphorylation of hexose. Microalgae are not able to metabolize the glucose under anaerobic conditions since not enough energy is obtained from the glucose and lactate dehydrogenase minority is slow down the process to complete the anaerobic fermentation. As Neilson and Lewin (1974) stated "probably the most notable difference in glucose metabolism in heterotrophic growth of microalgae, in comparison with autotrophic glucose metabolism or other non-carbohydrate organic substrates, is that under darkness, glucose is mainly metabolized via the PPP (pentose phosphate pathway)".

In summary, required concentration of glucose required for optimal growth should be known to define an exact conclusion. As the microalgal species and the environmental conditions are the key factors the reason may be related to combinations of those factors. Therefore, different consumption levels may be the result of combination of those. It is clearly understood that glucose can be localized as a preferred substrate for heterotrophic microalgae cultivation. Additionally, the enzymes catalyzing usage of other sources cannot be synthesized in the existence of the preferred substrate (Lewin and Hellebust, 1978).

1.5.4. Nitrogen metabolism

Nitrogen is exactly the most important element grant to the dry mass of microalgal cells up to 10% and nitrogen deficiency in microalgae metabolism results the accumulation of oils or polysaccharides (Kaplan et al., 1989). In addition, carbon and nitrogen metabolism are closely related in microalgae. They share carbon supplies in

autotrophic growth from the fixed CO_2 or comprehended organic carbon in heterotrophic growth. Additionally, energy from TCA cycle and mitochondrial electron transport chain is used (Inokuchi et al., 2002). Synthesize of the amino acids glutamate, glutamine and aspartate starts with the assimilation of ammonium and requires carbon skeletons like keto-acids and also ATP and NADPH (Huppe and Turpin, 1994). Supply of inorganic nitrogen limits the respiration rate according to solicitation for carbon skeletons and this occurs when intracellular carbohydrate energy reserves are accumulated. Nitrogen is a limiting factor when carbon is not (Geider and Osborne, 1989). Roughly, nitrogen has a well-seen positive effect on growth and a controlled effect on lipid accumulation within the microalgae metabolism. These organisms can comprehend different nitrogen sources, for example ammonia (NH_4), nitrate (NO_3) and urea. During our experiment different kind and amount of nitrogen source, diammonium tartrate and ammonium sulphate have been used. The results proved that the nitrogen limitation is enhancing the lipid accumulation in *Schizochytrium sp.* under specified conditions. It is obvious that biomass amount increases parallel to the nitrogen amounts. In this study diammonium tartrate and ammonium sulphate were used as nitrogen source and effects on biomass production and lipid accumulation of different amounts are examined for both sources. Furthermore, lots of nitrogen sources have been tested, in some of which the biomass amount is obtained high and for some the lipid content. Figure 3.4 represent some of the alternative nitrogen sources used for cultivation and resulting biomass-lipid amounts.

Table 1.2: Effect of different nitrogen sources on biomass and lipid amounts (Hsieh - Wu et al., 2009).

Nitrogen sources	Biomass ($g\,L^{-1}$)	Lipids ($g\,L^{-1}$)	Lipids in biomass (%, w/w)
Complex nitrogen			
Tryptone	2.133	0.505	23.68
Peptone	5.220	1.755	33.62
Yeast extract	6.108	2.063	33.77
Defined nitrogen			
Urea	2.725	1.183	43.39
Monosodium glutamate	6.585	2.195	33.33
Sodium nitrate	7.459	2.403	32.21
Ammonium chloride	6.230	1.223	19.62

Ammonium is the most energetically efficient source for the algae because less energy is required for intake of the molecule so it is the most preferred nitrogen source for these organisms (Wilhelm et al., 2006). Ammonium can be transported into the cells by a group of membrane proteins under autotrophic or heterotrophic conditions. One is affected by the nitrogen status of the cell has high affinity and the other exhibits linear affinity to the amount of nitrogen inside the cell (Howitt and Udvardi, 2000). Assimilation metabolism of ammonium is a two - step reaction and catalyzed by glutamine synthetase during both autotrophic and heterotrophic reactions. Glutamine synthetase produce glutamine first and then glutamate synthetase converts glutamine into two molecules of glutamate and one molecule of α-ketoglutarate (Vanoni and Curti, 2005). Following these reactions nitrogen is distributed to the amino acids to catalyze organic molecules inside the cells.

2. EXPERIMENTAL

2.1 Materials

Schizochytrium sp. microalgae was used for the production of biomass and oil. This organism was acquired from the American Type Culture Collection (ATCC), VA 20108, USA. The chemicals used for the preparation of complex and defined media were acquired from Merck and Sigma-Aldrich and all of these chemicals were analytically pure. Beckman Coulter Allegra X-14 series centrifuge was used for the separation and Nuve EV 018 Vacuum Dryer was used for drying the samples.

2.2 Methods

The biomass and oil production experiments were conducted as in figure 2.1 and the details were given as follows. Cells were grown in two step cultivation. Firstly, *Schizochytrium Sp* cells were cultivated in 100 ml complex medium for 48 hours under 25^{o}C and 200 rpm. Following this, the produced cells were inoculated to 100 ml defined medium and incubated for 48 hours under 25^{o}C, 200-rpm conditions. After 48 hours, the resulting biomass was subjected to Nile-Red staining to quantify the totail oil amount. To calculate the amount of obtained biomass, mediums were centrifuged at 2000 rpm to remove the defined medium content and to separate the biomass. Then this biomass was dryed with a vacuum dryer under 60 ^{o}C for 24 hours due to remove the resting moisture from the biomass.

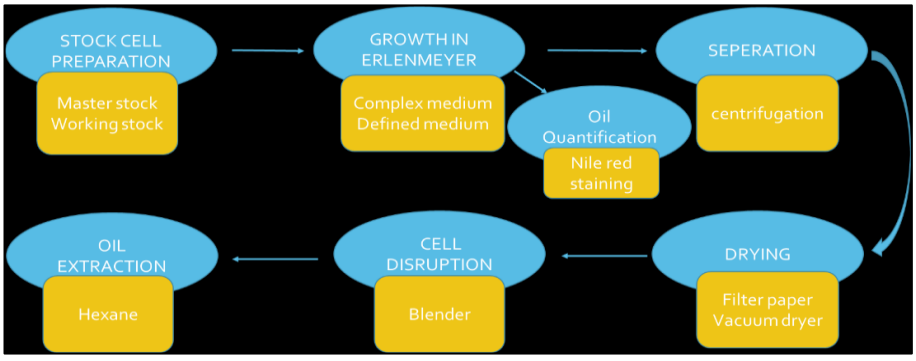

Figure 2.1: Flowchart showing the production process of biomass and total oil.

2.2.1. *Schizochytrium Sp.* stock cell preparation

1. 10 ml of 10 % DMSO appropriate medium was autoclaved and filtered sea water/ 70% of ethanol/ isopropanol/ were prepared in liquid nitrogen (Cryoprotectant solution). Prepared solution made filter sterilization was stored in an appropriate sterile tube.

2. As an alternative cryoprotectant solution, glycerol or methanol (%5-10) can be used. For DMSO and methanol 0,2 µm filter sterilization were made and for glycerol autoclave sterilization was performed.

3. Cell culture and cryoprotectant solution was mixed into sterile tubes with a ratio of 1:1.

4. For preparation of stock cell process, culture that is non-toxic, dynamic, relatively dense, end of the log phase or stationary phase at the beginning should be used.

5. Prepared homogeneous mixture was distributed into cryovials as 1 ml aliquots aseptically. Seed culture and working culture stock was prepared (figure 2.2). One of the master stock was used for preparing second master stock and rest of them was used for preparing study stocks. 1 ml of disposable study stock was planted for creating preliminary culture.

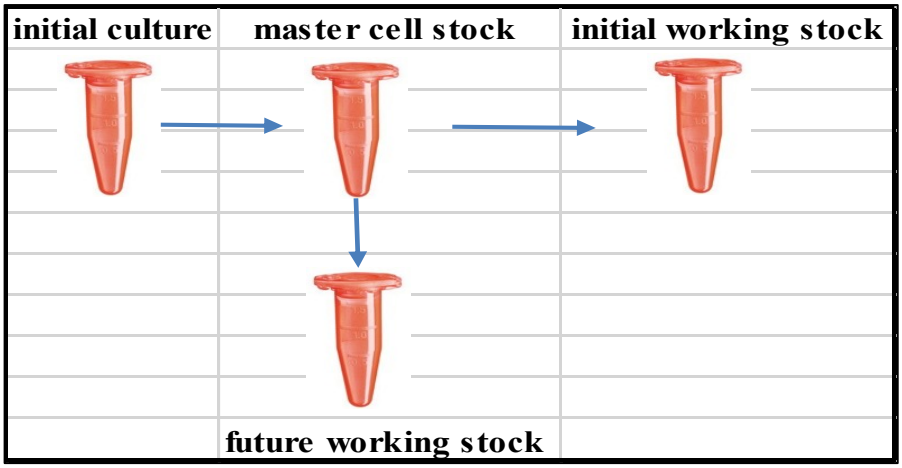

Figure 2.2: Two-stage preparation for master and working stock.

6. 250 ml isopropanol (C3H8O) was added into the Passive cooler/Mr. FrostyTM container. Cryovials were placed into the appropriate section of the passive cooler/Mr FrostyTM container.

7. Passive cooler/Mr FrostyTM container was placed at -80°C and was incubated 1-1.5 hours. Fridge (cabinet) at -80 must be kept closed during incubation.

8. After incubation, Mr FrostyTM container was removed and cryovials were placed into the liquid nitrogen tank immediately.

9. Process for placing and removing cryovials into the liquid nitrogen tank should be done as quickly as possible. Storage temperature should be <-130°C. Besides, special protective gloves must be used when using liquid nitrogen.

2.2.2. Cell culture preparation from main stock

1. To prepare cell culture, main cell stock was taken from liquid nitrogen tank and placed in the 40 ° C water bath to dissolve.

2. It is important that for many marine species incubation time at 40 ° C must not keep longer. Alternatively, longer incubation time at 25 ° C can be made but rapid warming dissolution method is more appropriate for stock cell.

3. Dissolved stock cells and necessary materials was transported under laminar flow conditions. Vial outer surface was cleaned with 70 % of ethanol,

4. High amount of bacterial or fungal spores can live in liquid nitrogen. Unopened vial should be cleaned with ethanol because there is a risk of contaminating culture for them.

5. 1 ml of dissolved main stock were transferred into 100 ml complex medium (table 2.1)

6. Prepared culture was incubated for 48 hours averagely at suitable temperature (20-25°C) and rpm (180-250).

2.2.3. Cell cultivation

1. 100 ml of complex medium was prepared and autoclaved.

2. The stocks were discarded from liquid nitrogen/ -80 fridge and then put into the 40°C water tank for 3-4 minutes to melt.

3. After the stock was completely melt 1 ml stock cell inoculated to the 100 ml of complex medium (Table 2.1) under sterile conditions.

4. For the availability test of stock cells 1/100 diluted amount was inoculated on agar petri dishes and after 48 hours the cell viability was controlled on plates.

Table 2.1: Complex and defined medium components.

Complex Medium				**Trace element solution(g/l)**	
Content	**g/l**	**g/l**	**g/l**	EDTA di-sodium salt	6
Glucose	40			FeCl3.6H2O	0,29
Peptone	8			H2BO3	6,84
Sea Salts	25			MnCl2.4H2O	0,86
MOPS	21			ZnCl2	0,06
Yeast Extract	5			CoCl2.6H2O	0,03
Defined Medium				NiSO4.6H2O	0,05
Glucose	80			CuSO4.5H2O	0
Diammonium tartrate Amonium Sulphate	1.65 1,18	2.0 1,47	2.4 1,70	Na2MoO4.2H2O	0,01
NaCl	12,50			**Vitamin Solution (mg/l)**	
$MgSO_4 \cdot 7H_2O$	2,50			Filter-sterilised (0.2 µm)	
KH_2PO_4	0.5			Thiamine	100
KCl	0.5			Biotin	0,5
$CaCl_2$	0.1			Cyanocobalamin	0,5
MOPS	21				
Trace elements*	5ml/l				
Vitamin solution*	1ml/l				

5. The inoculated medium was incubated for 48 hours on 250 rpm 25°C.

6. After 48 hours, 10 ml of these proliferated cells were inoculated to 3 different 100 ml defined medium. These samples were incubated on 250 rpm for 48 hours under 25°C. The initial pH of the culture was controlled with a buffer solution (potassium dihydrogen phosphate – sodium hydroxide) and adjusted to 7.0.

2.2.4. Biomass separation with centrifugation

1. After 48 hours incubation the growth cells were removed from incubator to centrifugation tubes in order to separate the produced biomass and the remaining medium.

2. Beckman Coulter Allegra X-15R centrifuge was used for separation process.

3. The process was implemented via 100 ml centrifugation tubes.

4. 2000 rpm centrifugation force was used and the supernatant part was discharged to obtain the biomass produced. And then the biomass was transferred to drying.

2.2.5. Drying of biomass

1. The centrifuged biomass was spread out on to the filter papers having 3 cm radius (Figure 2.3).

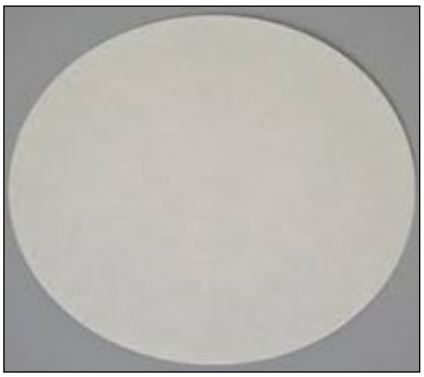

Figure 2.3: Filter Paper.

2. The amount of biomass deployed onto the filter papers are little and the layer spread was thin because of the efficient removal of humidity inside.
3. These filter papers containing biomass then exposed to 60˚C under 1 atm pressure in the Nuve EV 018 (Figure 2.4) model vacuum dryer to accelerate the evaporation and removal of moisture inside.

Figure 2.4: Nuve EV 018 Vacuum Dryer

4. Then the dried biomass (Figure 2.5) were stored for oil quantification.

Figure 2.5: Dryed biomass sample.

2.2.6. Nile red staining

1. The cell concentration was adjusted to 3 x 10^5 which means OD 0,6 under 750 um.
2. These concentration-adjusted cells were pretreated with %20 DMSO for 10 minutes at 25˚C.
3. 75 ul of those pretreated cells, 500 ul of %25 DMSO, 1420 ul of seawater and 5 ul nile red were mixed and kept for 10 minutes under 40˚C.
4. This last mixture was examined via a spectrofluorometer between 530 – 575 nm.

2.2.7. Cell disruption and hexane extraction

1. The dried biomass (Figure 2.5) was then subjected to cell disruption via a blender like machine.
2. Bosch MKM6000 (Figure 2.6) is used in order to decrease the amount of conglomerated particles and smash the cell wall.

Figure 2.6: Blender used to smash the biomass.

3. The grinded cells then was taken to hexane extraction step in order to determine the total oil amount.
4. Erlen Mayer flasks containing 1 gr biomass /10 ml hexane was prepared.
5. This mixture was blended for 6 hours and then the biomass residues filtered for the purpose of separation.
6. The last mixture containing hexane and oil was put in to a rotary evaporator to vaporize the hexane and to leave the oil it captured.
7. Lastly the volume of oil is measured.

3. RESULTS AND DISCUSSION

In this study, all the experiments were carried out to produce high amount of *Schizochytrium sp*. microalgae biomass and oil within this organism. Different nitrogen sources and amounts were examined during studies. Because one of the crucial parameter to produce high amount of oil is the optimization of nitrogen effect during manufacturing these organisms. According to results, both the source and amount of the nitrogen affects the biomass and oil amounts.

3.1. Nile Red Results for Ammonium Sulphate and Diammonium Tartrate

Table 3.1 shows the biomass amounts obtained with diammonium tartrate containing medium and Table 3.2 shows the results for ammonium sulphate containing medium. Results illustrates that diammonium tartrate more effective than ammonium sulphate to produce high amount of biomass since the amount of nitrogen supplied to media were the same. The reason of this could be noted as the level of the involvement of those nitrogen sources into TCA (Tricarboxylic Acid) cycle (James P. Wynn et al, 1999).

Table 3.1: Effect of diammonium tartrate on biomass amount
T: 25^{0}C, Mixing: 200 rpm, C source: Glucose syrup

Nitrogen Amount (g)	Biomass amount (g)
0,043	0,565
0,052	0,656
0,062	0,840

The amount of supplied nitrogen were same but the obtained biomass amount different probably due to the pH value of the medium during proliferation. Because diammonium tartrate provide ammonia and tartaric acid into the media and the reduced pH increase the adsorption of nitrogen (Perez-Garcia, O et al., 2011).

Table 3.2: Effect of Ammonium sulphate on biomass amount
T: 25^{0}C, Mixing: 200 rpm, C source: Glucose syrup

Nitrogen Amount (g)	Biomass amount (g)
0,043	0,255
0,052	0,281
0,062	0,357

Figure 3.1 compare the biomass amounts in a one graph for diammonium tartrate and ammonium sulphate. It is obvious that nitrogen amount have a pozitive effect on cell proliferation according to both results. Additionally, the effect of diammonium tartrate compare to ammonium sulphate is quite understandable because the organism could uptake more diammonium tartrate since the pH of the environment.

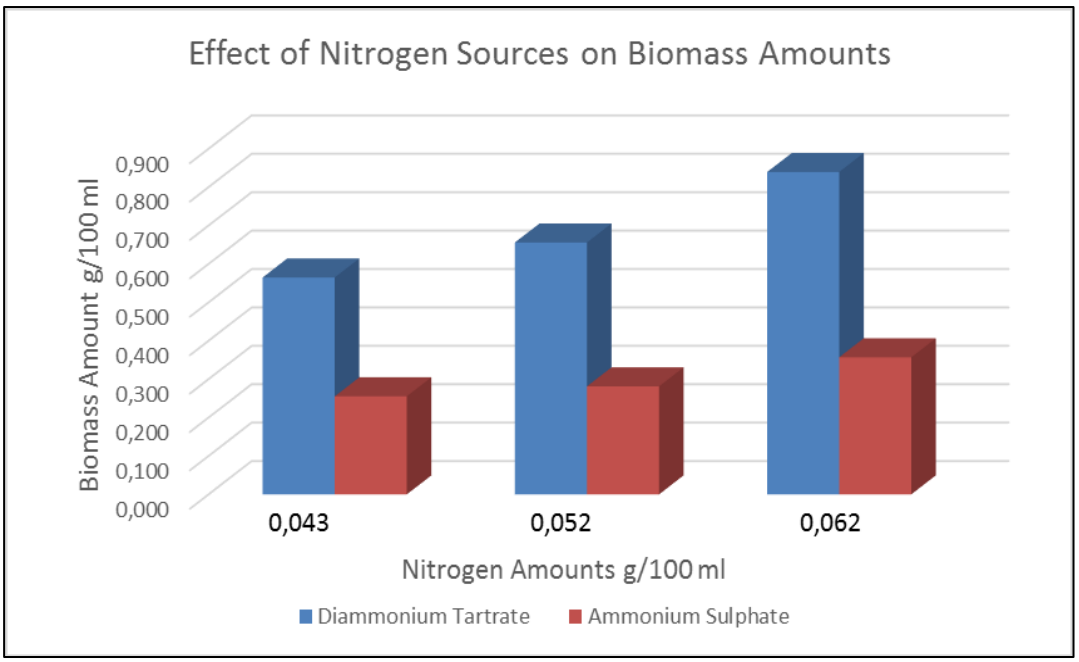

Figure 3.1: Biomass amounts for Diammonium Tartrate and Ammonium Sulphate.

Accumulation of lipids is generally associated with limitation of a key nutrient for organisms, usually nitrogen. Carbon uptake continues and accumulated as lipids after the nutrient becomes limited (Merzlyak et al., 2007). Below, Table 3.3 shows the oil amounts and percentages in produced biomass with diammonium tartrate containing medium and Table 3.4 shows the results for ammonium sulphate containing medium. Although there were no significance difference between the oil amounts for both nutrient, it could be noted that the percentage of the oils were high under less nitrogen conditions for both. As the growth media was including high amount of

glucose, results could be interpreted as *Schizochytium sp.* accumulate the excess amount of glucose as oil under nitrogen depletion.

Table 3.3: Effect of Diammonium Tartrate on oil produced in biomass
T: 25^{0}C, Mixing: 200 rpm, C source: Glucose syrup

Nitrogen Amount (g)	Oil amount (g)	% Oil
0,043	0,032	5,66
0,052	0,036	5,48
0,062	0,044	5,23

Also there is a different explanation of the mechanism for accumulation of lipids for different organisms. For example, in *Cryptheconidium conhii* and *C. sorokiniana* lipid accumulation may be independent from nitrogen limitation but related with the extreme amount of carbon in the media (Ratledge and Wynn, 2002).

Table 3.4: Effect of Ammonium Sulphate on oil produced in biomass
T: 25^{0}C, Mixing: 200 rpm, C source: Glucose syrup

Nitrogen Amount (g)	Oil amount (g)	% Oil
0,043	0,024	9,41
0,052	0,023	8,18
0,062	0,021	5,88

Results proved that an efficient production can be obtained if the exact nitrogen source is found and the amount is optimized under specified conditions during this study. Also, for the control of the other parameters, fermentor technology can be used to manufacture industrial scale production.

3.2. Hexane Extraction Results for Diammonium Tartrate Containing Medium

Table 3.5 shows the results for biomass amounts and Table 3.6 shows the total oil amounts for this biomass obtained via hexane extraction. As the results illustrates there were some difference for biomass amounts between Table 3.1 and Table 3.5 though all the parameters were the same. It was not such a significance difference so the results could be used to compare the total oil amounts for two different total oil quantification methods, hexane extraction and Nile red staining.

Table 3.5: Effect of diammonium tartrate on biomass amount

T: 25^{0}C, Mixing: 200 rpm, C source: Glucose syrup

Nitrogen Amount (g)	Biomass amount (g)
0,043	0,663
0,052	0,954
0,062	1,139

Table 3.6 shows the results for hexane extraction. The total oil amount can be seen on the second column and oil percantage on the third column. The percent of the oil with hexane extraction was less than the oil percent obtained via Nile red staining. As the total oil amounts did not differ sharply, the ratio between biomass and total oil differs as on Table 3.3 and Table 3.6

Table 3.6: Effect of Diammonium Tartrate on oil produced in biomass

T: 25^{0}C, Mixing: 200 rpm, C source: Glucose syrup

Nitrogen Amount (g)	Oil amount (g)	% Oil
0,043	0,024	3,80
0,052	0,029	3,03
0,062	0,040	3,51

These results showed that Nile red staining yield higher total oil compare to hexane extraction. The difference may be because of the hexane extraction efficiency. Additionally, Nile red method was used to quantify the total lipid amount inside the cell but hexane extraction yield the oil amounts inside the dried biomass.

Some additional studies could be designed based on these differences and it might be possible to develop a combinational quantification method for total oil measurement.

4. CONCLUDING REMARKS

The idea of producing omega acids from alternative sources arise from the fact that the current commercial origin of these lipids are globally marine fish. As it is obvious that fish stocks are declining day by day and this trend cannot afford the omega needs in a futuristic perspective. Indeed, fish cannot produce omega fatty acids; they get these fatty acids eating microalgae. Because of this basis, there have been many trials to maintain these molecules from cultivation of algae. For this purpose, heterotrophic microalgae have been used commercially to produce omega-3 fatty acids, especially DHA and EPA since 1950s (Figure 4.1).

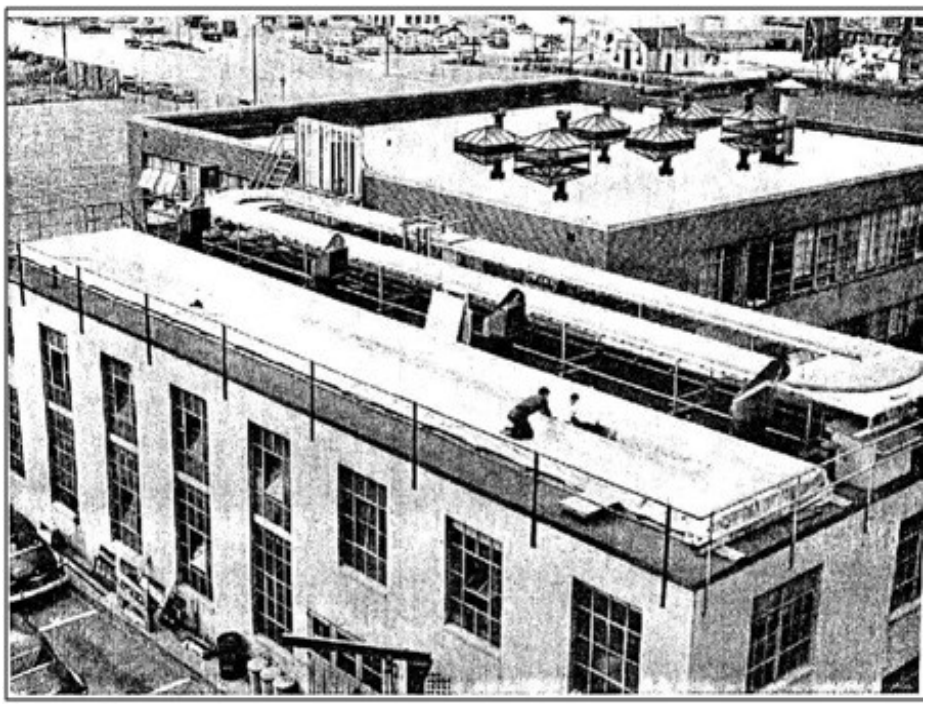

Figure 4.1: First algal mass culture experiment in MIT (Burlew, 1953).

Omega-3 and omega-6 are well known polyunsaturated fatty acids (PUFA) and they cannot be synthesized by our metabolism since they are essential for human body. Therefore, they must be obtained through our diet containing the omega products from animals, plants, fungi and microalgae (Ward and Singh, 2005). To prove the importance of fatty acids in our metabolism many studies have been carried out to

compare the eating habits between Mediterranean – Japanese style and fast foods (Gropper & Smith, J. (2012). For example, people who consume Japanese and Mediterranean food the Omega 6: Omega 3 ratio in blood close to 2:1 while people who consume fast food it is noted around 25:1 (Rubio-Rodriguez et al., 2010). In another study patients having cardiovascular diseases subjected to omega-3 fatty acids, mustard oil and placebo. After one year, the results showed that, the patients having omega and mustard oils had 25 % less new cardiovascular events (Kris – Etherton, 2002).

On the contrary, the availability of resources especially fish are highly fluctuating season to season so this badly effects the food chain, thus this reinforce the studies to discover alternative omega sources (Kalogeropoulos et al., 2010). Traditionally, fish oils were the main source for omega-3, but its usage has been limited due to problems such as unpleasant odour and taste, and poor oxidative stability (less shelf life). Furthermore, the presence of chemical contaminants in fish oil can be harmful to consumers (Aderma-Vega, 2012). Therefore, obtaining omega-3 from microalgae seems a logical, promising and eco-innovative solution.

Carbohydrate, protein and fats are basic intramolecular nourishments obtained from algae used to cover food needs and health. For example the average composition of proteins are 12-35%, fats are 7.2-23% and carbohydrates are 4.6-23% in microalgae and those nutritional products can be found in form of powders, tablets and capsules (Figure 4.2).

Microalgae	Producer	Product	Production (ton/year)
Spirulina	Hainan Simai Pharmacy Co. (China)	Powders, tablets, dan extract	3000
	Earthrise Nutritionals (USA)	Tablets, powders, drinks	
	Cynotech Corp. (USA)	Extract	
	Myanmar Spirullina Factory (Myanmar)	Tablets, chip, liquor, liquid extract	
Chlorella	Taiwan Chorella Manufacturinh Co. (Taiwan)	Tablets, powders, nectar, mie	2000
	Klotze (Jerman)	Powders	
Dunaliella salina	Cognis Nutrition and health (Australia)	Powders of β-caroten	1200
Aphanizoenon flos-aquae	Blue Green Foods (USA)	Capsules, crystal	500
	Vision (USA)	Powders, capsules	

Figure 4.2: Commercial Food Products from Microalgae (Gouveia et al., 2008).

Heterotrophic microalgae cultivation for production of commercially important molecules is an attractive choice because this has significant advantages like simplified cultivation process and cheap carbon sources (Tuchman, 2006). Additionally, new improvements in microalgae biotechnology covering heterotrophy, bioinformatics and genetic - metabolic engineering have come up (Boyle and Morgan, 2009). These genetic developments are also safe and adequate because microalgae cultivation might be managed getting over the risk of environmental contamination (Doebbe et al., 2007).

To conclude, the potential in algae technology and expansions will be illustrated via strategic approaches because entrepreneurs aware that the advantages of algae technology are limitless.

For further studies, different parameters such as C sources, nitrogen sources, oil extraction methods etc. may be investigated and optimized to produce high amount of *schizochytrium sp*. microalgae biomass.

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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

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